Endothelium-Dependent Vascular Relaxation Is Abnormal in the Coronary Microcirculation of Atherosclerotic Primates

Frank W. Sellke, MD, Mark L. Armstrong, MD, and David G. Harrison, MD

Atherosclerosis impairs endothelium-dependent relaxation of large conduit arteries. Because coronary resistance vessels are spared from the development of overt atherosclerosis, endothelium-dependent responses were examined in these vascular segments. Malaysian cynomolgus monkeys (n=6) were made atherosclerotic by being fed a 0.7% cholesterol diet for 18 months. Control monkeys (n=6) were fed a standard diet. Coronary microvessels (122–220 μm) were studied in a pressurized (20 mm Hg), no-flow state using a video-imaging apparatus. Relaxations of microvessels, preconstricted with the thromboxane analogue U46619, were determined in response to acetylcholine, bradykinin, the calcium ionophore A23187, adenosine, and sodium nitroprusside. Microvascular relaxations to bradykinin and A23187 were reduced in atherosclerotic monkeys compared with controls, whereas acetylcholine produced additional contraction in atherosclerotic monkeys. Responses of preconstricted microvessels to adenosine and sodium nitroprusside were identical in atherosclerotic and control animals. Indomethacin did not alter responses in control or atherosclerotic animals. Histologic examination revealed neither intimal thickening nor plaque formation in microvessels of this size class despite marked changes in conduit arteries. Electron microscopy showed minor alterations of endothelial cell morphology in microvessels of atherosclerotic animals. In conclusion, long-term hypercholesterolemia markedly impairs endothelium-dependent vascular relaxation in the coronary microcirculation where overt atherosclerosis does not develop. These changes in endothelial cell function may significantly alter regulation of myocardial perfusion by neurohumoral stimuli. (Circulation 1990;81:1586–1593)

The endothelium importantly regulates vascular smooth muscle tone in both conduit vessels and smaller resistance vessels.1–4 This regulatory function includes metabolism of vasoactive substances and the secretion of several vasoactive compounds. Some of the potent paracrine factors that may modulate vascular tone include prostacyclin and nonprostanoid relaxing factors and probably several constricting factors, including the endothelin peptide family.5

The nonprostanoid endothelin-derived relaxing factor (EDRF) is a short-lived compound or family of compounds that produce vascular relaxation by activating guanylate cyclase6,7 and hyperpolarizing vascular smooth muscle.8 Recently, it has become apparent that hypercholesterolemia and atherosclerosis markedly impair this important function of the endothelium in large conduit vessels.9–12 In these vessels, the endothelium is generally morphologically intact,13 although marked intimal atherosclerosis may be present. This abnormality appears to be predominantly related to the decreased release of EDRF.

While possibly important in the genesis of vascular spasm, this abnormality of conduit vessel function probably contributes little to the regulation of tissue perfusion in the absence of vascular spasm. Myocardial perfusion is regulated predominantly by resistance arteries less than 200 μm in diameter.14,15 The goal of the present study was to examine the effect of long-standing hypercholesterolemia on endothelium-dependent responses in the coronary microcirculation where gross atherosclerotic changes do not occur. A unique in vitro microvessel imaging apparatus was used that allowed vessels less than 200 μm to be studied. Responses of coronary microvessels from...
normal monkeys and monkeys fed a high cholesterol diet for 18 months were examined.

Methods

Adult male Malaysian cynomolgus monkeys were studied. Six monkeys were fed a commercial laboratory diet (Purina Monkey Chow, Ralston-Purina, Richmond, Indiana). Six monkeys were fed an atherogenic diet of 0.7% cholesterol for 18 months. At intervals of 3–4 months, the monkeys were sedated with ketamine hydrochloride (10 mg/kg i.m.), and a venous blood sample was obtained. Plasma cholesterol levels averaged 129±6 mg/dl in controls and 566±58 mg/dl in atherosclerotic monkeys. Monkeys weighed 6.4±0.4 kg in the control group and 5.8±0.4 kg in the atherosclerotic group (p=NS).

Vessel Preparation

Hearts were obtained at the conclusion of separately performed in vivo studies and stored in a physiological buffer solution overnight at 4°C. Coronary microvessels (122–220 μm in diameter and 3–4 mm in length) were dissected from diagonal branches of the left anterior descending coronary artery using a 40× dissecting microscope. Microvessels were placed in a Plexiglas isolated organ chamber, cannulated with dual glass micropipettes measuring 50–100 μm in diameter, and secured with 10-0 nylon monofilament suture. Kreb’s buffer, aerated with 95% O₂, 5% CO₂ and maintained at 36.5–37.5°C, was continuously circulated through the organ chamber. The maximum constriction to KCl (100 mM) was determined to occur at a mean distending pressure of 20 mm Hg. Vessels were, therefore, pressurized to 20 mm Hg in a no-flow state using a monometer filled with Kreb’s buffer. With a split-screen microscope connected to a video camera, the vessel image was projected onto a television monitor. A video electronic dimension analyzer (Living Systems Instrumentation, Burlington, Vermont) was used to measure lumen diameter and wall thickness (Figure 1). This device provides on-line digital display of vessel lumen diameter and a DC signal of lumen diameter, which is recorded. The use of this device to study the coronary microcirculation has been previously described. A pressure transducer measured distending pressure. Measurements were recorded with a Gould RS3000 strip-chart recorder (Gould, Cleveland, Ohio). Vessels were allowed to bathe in the organ chamber for 60 minutes before any interventions.

Study Protocol

Coronary microvessels were preconstricted with 1–100 nM U46619 (a stable thromboxane A₂ analogue) by 30–70% of the baseline diameter. After stabilization of the constricted diameter, drugs were applied extraluminally.

In each vessel, responses to two to four vasorelaxing substances were examined. The order of drug administration was randomized for acetylcholine, bradykinin, sodium nitroprusside, and adenosine. Responses to the calcium ionophore A23187 were always examined last. Vessels were washed with Kreb’s buffer and allowed to equilibrate for 15 minutes between interventions. Two or three vessels were examined from each animal. The response to any given agonist was only examined once for each animal studied. Measurements were always made 2–3 minutes after the drug was administered, when the response had stabilized.

In some experiments, endothelium-dependent relaxations were examined in the presence of 10 μM indomethacin to inhibit the production of cyclooxygenase products (n=2 for control and 2 for atherosclerotic).

Endothelial Function

To directly assess the role of the endothelium in mediating responses to bradykinin and acetylcholine, the endothelium was selectively damaged in two normal microvessels. To accomplish this, saponin (approximately 0.2 ml of a 200 μg/ml solution) was administered intraluminally over 20–30 seconds. After this, the lumen was flushed thoroughly with Kreb’s buffer. In these experiments, responses to acetylcholine, bradykinin, and sodium nitroprusside were examined.
Histology

Sections of myocardium were fixed in formalin (2%) and examined using hematoxylin and eosin or Van Gieson’s staining. Additional sections were fixed in 2% glutaraldehyde in 0.1 M cacodylate (pH=7.25). Longitudinal specimens were cut from each myocardial section postfixed in osmium tetroxide and coated with carbon and gold-palladium alloy. These sections were examined with scanning electron microscopy. Selected specimens were postfixed in osmium tetroxide and potassium ferrocyanide, and thin-sectioned (600–700 Å) sections were mounted on a 200-mesh copper grid and stained with uranyl acetate and lead citrate. These sections were examined with transmission electron microscope.

Drugs

Acetylcholine-HCl, bradykinin, calcium ionophore A23187, adenosine, saponin, and sodium nitroprusside were obtained from Sigma (St. Louis, Missouri). The thromboxane mimetic U46619 was obtained from Upjohn (Kalamazoo, Michigan). Solutions of acetylcholine, bradykinin, adenosine, and sodium nitroprusside were prepared in ultrapure water. Solutions of saponin were prepared in Kreb’s buffer. The calcium ionophore A23187 was mixed in dimethylsulfoxide to make a 10^{-2} M stock solution that was kept at −20°C. The thromboxane analogue U46619 was mixed in ultrapure water to make a 10^{-2} M stock solution that was also kept at −20°C. Indomethacin was mixed in saline (0.9%) and adjusted with NaOH to pH 7.8 to make a 10^{-3} M stock solution. Dilutions were prepared daily.

Statistical Analysis

Responses of coronary microvessels to the various drugs were compared using analysis of variance. Whenever significance was indicated the Schéffe’s F test was used to compare between groups. Statistical significance was considered present when p<0.05. Values were reported as mean±SEM.

Results

The average vessel size in the control group was 180±141 and 169±111 μm in the atherosclerotic group. The degree of preconstriction was similar in vessels from two groups of monkeys (56±3% and 47±4% of the baseline diameter in atherosclerotic and control groups, respectively). The average concentration of U46619 required to achieve this constriction was significantly greater in the control group (log molar, −6.84±0.17) versus the atherosclerotic group (log molar, −8.08±0.24) (p<0.01). Vessels preconstricted with U46619 maintained a constant diameter for more than 40 minutes if no relaxing agent was applied.

Endothelium-Dependent Responses

Relaxation to bradykinin of coronary microvessels from atherosclerotic monkeys was markedly reduced compared with those from control monkeys (Figure 2). Acetylcholine administered to preconstricted vessels from atherosclerotic monkeys produced minimal relaxation or constriction compared with controls (Figure 3). The calcium ionophore A23187, which releases EDRF through non-receptor-mediated mechanisms, also had markedly diminished effects on microvessels from atherosclerotic monkeys (Figure 4).

Endothelium-Independent Responses

Sodium nitroprusside, which relaxes vascular smooth muscle by activation of guanylate cyclase through an endothelium-independent mechanism, produced identical responses in microvessels from atherosclerotic and control monkey hearts (Figure 5). Adenosine, which also relaxes vascular smooth muscle through an endothelium-independent mechanism, produced nearly identical relaxation in microvessels from atherosclerotic and control monkeys (Figure 6).
Effect of Indomethacin

Indomethacin (10⁻⁵ M) had minimal effect on relaxation of coronary microvessels to acetylcholine and the calcium ionophore A23187 (Table 1). The relaxation to bradykinin was slightly reduced compared with control vessels not exposed to cyclooxygenase inhibition (Table 1). The reduced relaxation seen in microvessels from atherosclerotic monkeys to bradykinin and calcium ionophore A23187 were not substantially altered in the presence of indomethacin (Table 1). Mean constriction in atherosclerotic microvessels to acetylcholine was also not substantially altered (Table 1).

Endothelial Removal With Saponin

The intraluminal infusion of saponin (200 μg/ml×20-30 seconds) in normal vessels markedly reduced relaxations to acetylcholine (peak relaxation, 14%) and bradykinin (peak relaxation, 10%). In contrast, relaxations to sodium nitroprusside were not significantly altered (maximum relaxation, 71%; log ED₅₀ -5.1). These findings are consistent with the concept that relaxations induced by acetylcholine and bradykinin are due to the release of the EDRF, whereas relaxation to sodium nitroprusside is due to the direct effect of this substance on coronary vascular smooth muscle.

Histologic Assessment of Atherosclerosis

After fixation with formalin, sections of myocardium containing coronary microvessels stained with hematoxylin and eosin and van Gieson’s stain showed no gross or microscopic abnormalities. Specifically, there was no evidence of plaque or intimal thickening in the size class vessel used in this study. Large coronary arteries showed marked gross and microscopic atherosclerotic changes in all cases examined (Figure 7).

Electron Microscopic Findings

Scanning and transmission electron microscopy revealed an intact endothelial layer in atherosclerotic microvessels. On transmission electron microscopy, occasional endothelial intracellular vacuoles were apparent. The vascular smooth muscle appeared normal. No intimal thickening or gross plaque formation was observed (Figure 8B).

Discussion

Previous studies of large conduit vessels have clearly established that endothelium-dependent vascular relaxation is abnormal in the setting of atherosclerosis.⁹⁻¹² This appears predominantly related to abnormal production of EDRF rather than to de-

Table 1. Responses in the Presence of Indomethacin (10⁻⁵ M)

<table>
<thead>
<tr>
<th></th>
<th>Acetylcholine</th>
<th>Bradykinin</th>
<th>A23187</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Relaxation (max %)</td>
<td>100</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>ED₅₀ (log molar)</td>
<td>-5.5</td>
<td>-7.3</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>Relaxation (max %)</td>
<td>-13*</td>
<td>0.0</td>
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<tr>
<td></td>
<td>ED₅₀</td>
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*Negative values indicate constriction.
creased sensitivity of the vascular smooth muscle to the EDRF. The present experiments show that endothelium-dependent relaxation is also strikingly abnormal in the coronary microcirculation of cholesterol-fed primates. These findings have implications regarding regulation of myocardial perfusion in the setting of atherosclerosis, insight regarding the mechanisms underlying abnormal endothelium-dependent responses in atherosclerosis, and the question of whether atherosclerosis versus hypercholesterolemia per se may alter endothelium-dependent responses.

**Implications Regarding Myocardial Perfusion**

The vessels examined in the present study represent true coronary resistance vessels. This conclusion is based on observations made by Chilian et al and Nellis et al, who have shown that up to 50% of coronary resistance resides in coronary microvessels between 100 and 200 μm in diameter. These original studies were performed in rabbits and cats. More recently, Chilian et al examined the coronary microcirculation in the coronary circulation of the cynomolgus monkey, the animal model used in the present study. In those in vivo studies, vessels from 200 to 300 μm in diameter contributed approximately 25% to total coronary resistance. Therefore, our present findings suggest that both humoral and neuronally released substances, which elicit the release of the EDRF (e.g., acetylcholine, serotonin, norepinephrine, or vasopressin), may have either blunted or perhaps disparate effects in atherosclerotic coronary resistance vessels compared with controls. The findings indicate that neurohumoral regulation of myocardial perfusion may be abnormal in the setting of hypercholesterolemia and atherosclerosis.

*Mechanisms Underlying Abnormal Endothelium-Dependent Vascular Relaxation in Atherosclerosis*

Several explanations have been provided for abnormal endothelium-dependent responses in atherosclerotic vessels; these include decreased or abnormal production of the EDRF, destruction of the EDRF due to an intimal barrier, the concomitant release of a constricting factor, and abnormalities of endothelial cell membrane receptor-second messenger interactions. In our present study, it is unlikely that an intimal barrier played a substantial role because the intima was not thickened in the size of coronary microvessel studied. It is also unlikely that this defect in endothelial function is only related to abnormalities of endothelial cell receptor–second messenger interactions. This is because the calcium ionophore A23187 (which releases the EDRF via non-receptor mechanisms) also failed to elicit endothelium-independent relaxation in coronary microvessels of...
Figure 8. A: Scanning electron micrograph of atherosclerotic coronary microvessel (×1,000). B: Transmission electron micrograph of atherosclerotic coronary microvessel (×3,000). Both scanning and transmission electron microscopy show the endothelial layer to be intact. No intimal thickening is present, but occasional endothelial vacuoles are observed.

Atherosclerotic monkeys. We cannot exclude the possibility that abnormal endothelium-dependent responses in this setting are due to the concomitant release of a non-prostanoid-constricting factor.

It has been debated whether hypercholesterolemia per se or the atherosclerotic process is responsible for abnormal endothelium-dependent responses observed in studies of large vessels. Although the vessels studied in the present experiments did not develop overt atherosclerosis, vacuoles likely representing lipid droplets could be seen within the endothelium on transmission electron microscopy. It is conceivable that the accumulation of lipids with associated oxidative processes may be in large part responsible for decreased production and/or increased intracellular destruction of EDRF. Thus, the incorporation of lipids within the endothelium, an early manifestation of atherosclerosis, may have contributed to this abnormality in the coronary microcirculation. Other mechanisms likely contribute to this abnormality. It is possible that activated macrophages within the upstream large coronary arteries released substances that affect the downstream endothelial cells.

The present experiments confirm work by Yamamoto and coworkers,19 who have shown that endothelium-dependent responses are abnormal in the microvessels (<25 μm) of the cremaster in rabbits with diet-induced atherosclerosis. More recently, Osborne and coworkers20 have shown that small diagonal branches of the left anterior descending artery removed...
from the surface of rabbit hearts fed a high cholesterol diet exhibit abnormal endothelium-dependent vascular relaxation compared with normal hearts. Our study extends these observations by showing that substantially lower (but still quite elevated) cholesterol levels can alter microvascular responses. Furthermore, these studies were performed in a primate model of atherosclerosis with histological similarities to that of human atherosclerosis. Lastly, we have examined responses to several endothelium-dependent vasodilators, including the calcium ionophore that elicits the release of EDRF via non-receptor-mediated mechanisms.

**Advantages and Limitations**

The experimental preparation used has several unique advantages over other approaches to studies of the coronary microcirculation. This preparation allows direct examination of changes in vascular diameter in vitro in response to a variety of vasoreactive agents. Because of the in vitro nature of the study, vascular distending pressure can be precisely regulated and does not vary despite large changes in vessel diameter that may occur during active vasomotion. This approach to in vitro studies of microvessels minimizes instrumentation of the vascular lumen and inadvertent damage to the endothelium. Because of the in vitro nature of the study, direct inference cannot be made regarding regulation of myocardial perfusion; however, in several other instances, findings in vitro have paralleled findings in vivo.4,21–25

In the present study, endothelial damage produced by the brief infusion of the detergent saponin abolished relaxations to acetylcholine and bradykinin. This was not a nonspecific effect of saponin because relaxations to sodium nitroprusside and constriction to the thromboxane mimetic U46619 were not affected. These studies prove that the endothelium must be intact for acetylcholine and bradykinin to produce vascular relaxation in coronary microvessels of this size.

To examine vascular relaxation, vessels were preconstricted with the thromboxane mimetic U46619. In preliminary studies, this agent was found to achieve stable baseline tensions that could be sustained for more than one half hour. The concentration of U46619 necessary to achieve equivalent degrees of preconstriction was greater for the normal vessels than the atherosclerotic vessels. While the explanation for this is unclear, it is possibly related to the basal release of EDRF, which is likely depressed in microvessels from atherosclerotic animals. Thus, the action of the thromboxane analogue was unopposed in the vessels from the atherosclerotic group. This unlikely artifactually affected the results of the study for two reasons. First, in general, higher concentrations of preconstricting substances tend to decrease responses to vasorelaxants. Second, relaxations to sodium nitroprusside and adenosine, which do not require an intact endothelium, were similar between normal and atherosclerotic vessels. Had the higher concentration of the thromboxane analogue used in the normal group in some way nonspecifically enhanced vascular relaxations, responses to these agents would have been expected to be similarly affected.

Although controversial, adenosine has been implicated as one mediator of metabolic and autoregulatory control of myocardial perfusion and the reactive hyperemic response. Because responses to adenosine were not altered in vessels from atherosclerotic animals, it might be inferred that responses to adenosine released endogenously during metabolic or autoregulatory adjustments of myocardial perfusion would be normal in hypercholesterolemia and atherosclerosis. Indeed, we have found changes of perfusion in response to sudden metabolic stress to be normal in atherosclerosis.26

In summary, the present experiments show that in addition to altering endothelial regulation of vascular tone in large vessels, long-standing hypercholesterolemia markedly impairs endothelium-dependent vascular relaxation in the coronary microvasculature. The role of this abnormality in the pathogenesis of myocardial ischemia remains to be determined; however, it is conceivable that impaired endothelial function within the microcirculation may contribute to abnormalities of myocardial perfusion. Because the EDRF has also been recognized to have important antithrombotic effects, this condition may as well contribute to small vessel platelet aggregation.27

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**References**

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